I hereby certify that this correspondence is being deposited with the United States Patent and Trademark Office via EFS **March 31, 2009**.

3/31/09 Michelle Hobson
Date Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Examiner:
Baltimore *et al.*	Delia RAMIREZ
Application No. 10/656,531	Group Art Unit: 1652
Filed: September 5, 2003	Confirmation no.: 8769
For: USE OF CHIMERIC NUCLEASES TO STIMULATE GENE TARGETING	

BRIEF ON APPEAL UNDER 37 C.F.R. § 41.37

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

An Appeal Brief was filed March 2, 2009 pursuant to 37 C.F.R. § 41.37 (see, Fed. Reg. vol. 73. no. 238, page 74972 published December 10, 2008) in response to the Final Office Action mailed November 14, 2008, the Advisory Action mailed January 9, 2009 and a Notice of Appeal was received in the USPTO on January 27, 2009.

A Notice of Non-Compliant Appeal Brief was mailed on March 26, 2009, noting that canceled claim 106 was cited in the Grounds of Rejection and in the Argument headings. Thus, pages 4-11 of the Brief are resubmitted herewith.

I. REAL PARTY IN INTEREST

California Institute of Technology is the assignee of record, based on an assignment from the inventors recorded on November 8, 2004 at Reel 015343, Frame 0951. In addition, a confirmatory license from California Institute of Technology to National Institutes of Health, U.S. Department of Health and Human Services, U.S. Government was recorded on July 29, 2008 at Reel 021307, Frame 0788. Thus, California Institute of Technology and the U.S. Government are the real parties in interest.

II. RELATED APPEALS AND INTERFERENCES

Appellants are not aware of any related appeals or interferences.

III. STATUS OF CLAIMS

Pending: Claims 21, 28, 40, 43, 99 to 104, 107 to 113, 120 to 135 and 137 to 143

Canceled previously: Claims 1 to 20, 22 to 27, 29 to 39, 41, 42, 44 to 98, 105, 106, 114 to 119 and 136

Canceled by amendment herein pursuant to 37 CFR § 41.33(b): Claim 40

Withdrawn: Claims 43, 109 to 113, 120 to 135 and 137 to 143

Rejected: Claims 21, 28, 40, 99 to 104, 107 and 108

Appealed: Claims 21, 28, 99 to 104, 107 and 108

IV. STATUS OF AMENDMENTS

The amendments made in the paper responsive to the Final Office Action were entered for purposes of Appeal by the Examiner. (Advisory Action, Box 7).

In addition, pursuant to 37 C.F.R. § 41.33(b)(1), Appellants request cancellation of claim 40, which is essentially duplicative of appealed claim 28. Cancellation of claim 40 does not affect the scope of any other appealed claim and is therefore proper on appeal.

Thus, the claims on appeal are as shown in the attached Claims Appendix.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Independent claim 21 is drawn to a vector comprising a nucleic acid encoding a chimeric nuclease and a nucleic acid comprising a repair substrate (page 8, lines 15-22). The chimeric nuclease comprises: (i) a zinc finger DNA binding domain; (ii) a cleavage domain; and (iii) a nuclear localization signal (page 8, lines 15-22; page 9, lines 1-2). The repair substrate comprises (i) a nucleic acid sequence that is substantially identical to a region flanking a target sequence in chromosomal DNA; and (ii) a nucleic acid sequence which replaces the target sequence upon recombination between the repair substrate and the target sequence (page 8, lines 15-22).

Independent claim 28 is drawn to an isolated mammalian cell comprising (a) a chimeric nuclease comprising a zinc finger DNA-binding domain and a cleavage domain; and (b) a repair substrate comprising (i) a nucleic acid sequence that is substantially identical to a region flanking a target sequence in endogenous chromosomal DNA; and (ii) a nucleic acid sequence which replaces the target sequence upon recombination between the repair substrate and the target sequence (page 9, lines 3-12; page 10, lines 14-15; page 11, lines 1-9).

Claim 99 depends from claim 21 and further indicates that the nucleic acid encoding the chimeric nuclease is operably linked to a promoter (page 8, lines 23-14).

Claim 100 depends from claim 99 and further indicates that the promoter is an inducible promoter (page 8, lines 25-26).

Claim 101 depends from claim 99 and further indicates that the vector is a viral vector (page 8, line 26).

Claim 102 depends from claim 21 and specifies that the vector further comprises a nucleic acid encoding a second chimeric nuclease, wherein the second chimeric nuclease forms a heterodimer with said chimeric nuclease (page 10, lines 20-23).

Claim 103 depends from claim 28 and specifies that the chimeric nuclease is encoded by a nucleic acid that is operably linked to a promoter (page 9, lines 18-19).

Claim 104 depends from claim 103 and further specifies that the promoter is an inducible promoter (page 9, lines 19-20).

Claim 107 depends from claim 28 and further specifies that the cleavage domain comprises a cleavage domain of a type IIs restriction endonuclease (page 7, lines 21-23).

Claim 108 depends from claim 107 and further specifies that the cleavage domain comprises a FokI cleavage domain (page 7, lines 21-23).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Whether claims 21, 28, 99-104, and 107-108 are unpatentable under 35 U.S.C. § 103(a) as obvious over U.S. Patent Publication No. 20020107214 (hereinafter "Choulika") in view of Bibikova et al. (2001) *Mol. Cell. Biol.* 21:289-297 (hereinafter "Bibikova") and further in view of Takeuchi et al. (2002) *Biochem. Biophys. Res. Commun.* 293:953-957 (hereinafter "Takeuchi")

VII. ARGUMENTS

A. Claims 21, 28, 99-104, and 107-108 are not obvious over the cited references

Claims 21, 28, 99-104, and 107-108 remain rejected under 35 U.S.C. § 103(a) as allegedly obvious over Choulika in view of Bibikova and further in view of Takeuchi. (Final Office Action, paragraphs 10-12 and Advisory Action, pages 2-6). With respect to the vector claims (claims 21 and 99-102), the Examiner asserts that while Choulika, Bibikova and Takeuchi fail to teach the repair substrate and nuclease on the same vector, it would somehow be obvious to the skilled artisan from the general common knowledge to prepare vectors as claimed. *Id.* Likewise, with respect to the claims directed to cells comprising a chimeric nuclease (claims 28, 103, 104 and 107-108), the Examiner continues to assert that the references and state of the field somehow suggest binding of a chimeric nuclease to an endogenous chromosomal target site. *Id.*

Appellants address these points in turn.

(1) Vectors carrying both a coding sequence (chimeric nuclease) and repair substrate sequence are non-obvious over the cited references

Appealed claims 21 and 99-102 are drawn to vectors require that both a sequence encoding the chimeric nuclease and a repair substrate sequence are carried on the same vector. As repeatedly noted by Appellants, the references teach that coding sequences and repair (non-coding) sequences should be separated onto separate vectors. The separation of coding and non-coding sequences evidently ensures that there is no interference between the two sequences.

However, the Examiner has continued to assert that one of skill in the art would actually see it as simpler (and therefore beneficial) to include coding and non-coding sequences on the same vector. In response to Appellants noting that absolutely no evidence has been presented supporting this assertion, the Examiner stated (Advisory Action, paragraph 4):

> However the Examiner strongly disagrees with applicant's contention that one of skill in the art would not have recognized the advantages of delivering a single vector instead of two without reading applicant's specification because, as extensively discussed in the previous Office actions, it is essentially simpler to deliver one vector instead of two to a cell.

Again, no evidence was presented in support of the assertion that a single vector is "simpler." Indeed, the "extensive" discussion in the previous Office Actions includes admissions that the references fail to teach the claimed vectors along with continually unsupported assertions about what would be allegedly be "beneficial" (Final Office Action, paragraph 12):

> The Examiner acknowledges the amendments to the specification and agrees that none of the references specifically teach a single vector comprising the recited nucleic acids. ... However, the Examiner disagrees with Applicant's contention that the claimed invention is not obvious over the cited references. While it may be that in some instances the use of two vectors as opposed to a single one would be advantageous, as in indicated in

> the previous Office action, one of the skill in the art would be motivated to use a single vector for the benefit of delivering to the cell the necessary components for recombination a single vehicle.

In this discussion, there was still not one reason given in support of the supposed benefits of using a single vector as claimed. The fact remains that the skilled artisans working in the field of homologous recombination specifically chose to separate the coding sequences and the non-coding sequences onto different vectors because they believed it would be beneficial and simpler to keep them separate. It is clear that they did not want expression of the nuclease to interfere with access to the repair substrate or vice versa.

Thus, the evidence establishes that the skilled artisan would have thought including both the coding sequence and non-coding sequence on the same vector would complicate matters and be detrimental to their individual functions. Accordingly, there is absolutely no motivation or expectation of success from references that admittedly chose to separate coding and non-coding sequences onto separate vectors that the claimed vectors would be "simpler" or "beneficial." Simply stated, if it was obvious to the skilled artisan to use a single vector as alleged by the Examiner, the artisans in the field at the time of filing would have used such a single vector. These artisans however, did not because there was no expectation that such a vector would be functional.

As repeatedly pointed out, an obviousness rejection is only proper when the proposed combination of elements results in a predictable outcome (see, Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 in view of the Supreme Court Decision in *KSR International Co. v. Teleflex Inc.*, Fed. Reg. Vol. 72, No. 195, October 10, 2007, emphasis added):

> The rationale to support a conclusion that the claim would have been obvious is that all the claimed elements were known in the prior art and one skilled in the art could have combined the elements as claimed by known methods with no change in their respective functions, and the combination would have yielded nothing more than predictable results to one or ordinary skill in the art at the time of the invention.

Here, the evidence establishes that combining the chimeric nuclease coding sequence and repair substrate sequence onto a single vector is **un**predictable in terms of expression of the coding sequence and/or availability of the repair substrate. Thus, the rejection to claims 21 and 99-102 cannot be sustained.

(2) Mammalian cells comprising repair substrates that are homologous endogenous chromosomal DNA are non-obvious over the cited references

Appealed claims 28 and 103, 104, 107 and 108 are drawn to isolated mammalian cells comprising a chimeric nuclease and a repair substrate that includes sequences that are homologous to endogenous chromosomal DNA. In other words, Appellants' nucleases are cleaving endogenous chromosomal DNA and stimulating homologous recombination at endogenous sites. It is admitted that by the Examiner that the references fail to teach zinc finger proteins that recognize endogenous chromosomal sequences (Advisory Action, paragraph 6):

> ...[N]either Choulika et al. nor Bibikova et al. explicitly teach engineering of zinc finger proteins to recognize endogenous chromosomal sequences...

Nonetheless, in response to Appellants' arguments that there is no combination of references that teach or suggest cells as claimed (homologous recombination in endogenous chromosomal DNA), it has repeatedly been alleged that engineering zinc finger protein transcription factors to modulate expression of endogenous genes as taught in the art and that it would have been obvious to apply these teachings to chimeric nucleases (Advisory Action, paragraph 7):

> As previously discussed, it is clear from the teachings of Choulika et al. and Bibikova et al. that the purpose of introducing the SceI target site in the chromosome was to demonstrate the principle of targeting the chromosome of a host cell for repair. Therefore, the "endogenous" limitation is at a minimum suggested by the cited references and teachings of the prior art as cited by Applicant would have provided one of the skill in the art with the knowledge required to engineer the zinc finger DNA binding domain to recognize an endogenous chromosomal target.

It remains the case that obviousness cannot be established by showing that zinc finger proteins can be engineered to bind to chromosomal targets. Rather, as the Supreme Court in *KSR* reiterated that an obviousness inquiry is fact-dependent and that "a patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." *KSR*, 82 UPSQ2d at 1389.

Thus, the issue is not whether engineered zinc finger proteins were known to bind to endogenous genes. The issue is whether it was, at the time of filing, predictable from Choulika and/or Bibikova that chimeric nucleases comprising engineered zinc finger proteins and a cleavage domain would cleave endogenous DNA such that homologous recombination with that endogenous DNA was stimulated.

In fact, the evidence of record establishes that cleavage of non-endogenous chromosomal targets is not at all predictive of cleavage of endogenous chromosomal targets as claimed.

This evidence includes the teachings of the as-filed specification, which shows it was clearly not an established use of zinc finger nucleases to cleave endogenous chromosomal targets (see, paragraph [0160] of the as-filed specification, emphasis added):

> In the GFP gene targeting system the introduction of a DSB stimulated GT by >2000-fold and the absolute rate of gene targeting reached 3-5% when conditions were optimized. Such a system, however, depended on the prior introduction of a Sce site into the target gene and therefore **can not** be used for endogenous genes. To stimulate gene targeting at endogenous genes, a method to create sequence specific DSBs in those genes needs to be developed.

Simply put, the specification evidences that a foreign sequence inserted into a chromosome was not considered an endogenous target and that it was certainly not considered predictable at the time of filing that zinc finger nucleases would cleave endogenous chromosomal targets.

The cited references also fail to establish predictability of cleaving endogenous target sites with chimeric nucleases. Indeed, the primary reference, Choulika, clearly teaches that an endogenous chromosomal target is different than an inserted chromosomal target for the naturally occurring SceI DNA-binding domain. Choulika is clear that the

SceI binding site is not endogenous to a mammalian genome and thus must be inserted for cleavage to occur (see, Choulika, paragraph [0025], emphasis added):

> [0025] A model chromosomal loci was generated in which a site for the meganuclease I-SceI was introduced within the target region for recombination, and double stranded DNA cleavage via introduction of a vector encoding the restriction endonuclease was induced.

As is well known to the skilled artisan, endogenous cellular DNA is packaged in chromatin and it is entirely unpredictable to the skilled artisan from Choulika's introduced target sites that are likely not packaged into chromatin whether nucleases comprising engineered zinc finger proteins would actually cleave endogenous targets. Furthermore, as Choulika pre-dates all the cited art regarding zinc finger protein engineering, it is clear that this reference does not establish predictability of using chimeric zinc finger nucleases to cleave endogenous chromosomal DNA.

Similarly, Bibikova fails to teach cleavage of an endogenous chromosomal target in a mammalian cell. Instead, this reference specifically teaches that the target sequence for the naturally occurring ZFN is "microinjected" into frog oocytes (see, Bibikova, page 290, paragraph bridging left and right columns, emphasis added; see, also, Fig. 1(B) showing that target DNA is injected into oocytes):

> Here we characterize the cleavage abilities of the chimeric nuclease in *Xenopus laevis* oocytes. These enormous cells have a large capacity for homologous recombination that is readily accessed by microinjection of appropriate substrates ...

In fact, Bibikova clearly chose not to cleave endogenous chromosomal DNA because they were unsure if zinc finger nucleases would result in targeting of endogenous chromosomal DNA by a repair substrate (see, Bibikova page 296, right column, emphasis added):

> Several additional issues remain to be addressed to confirm the utility of chimeric nucleases as tools for gene targeting. Among these are demonstrating discrimination against related sequences; proving the

> efficacy of zinc fingers designed to bind arbitrarily chosen sequences; and testing the cleavage of genuine **chromosomal** targets.

Thus, like Choulika, Bibikova fails to teach cleavage of endogenous target site. In fact, even in light of the art regarding engineered zinc finger proteins available when Bibikova was published, this reference still teaches that it was not considered predictable to induce homologous recombination at an endogenous chromosomal target sequence cleaved with a chimeric nuclease, as claimed.

Still further evidence that cleavage of inserted/injected target sites is not predictive of cleavage of endogenous target sites as claimed is found in Porteus et al. (2005) *Nature Biotechnology* 23:967-973 (Evidence Appendix (1) attached hereto). This paper, published well after Choulika, Bibikova and the earliest priority date of the instant application, clearly indicates that, even after the instant specification was filed, zinc finger nuclease mediated cleavage of endogenous chromosomal target sites in mammalian cells was not predictable from Choulika's inserted SceI sites (Porteus, Box 3 on page 969):

> Although several hundred different homing endonucleases with different recognition sites have been identified, the major limitation to using them in gene targeting is that most mammalian genes do not have recognition sites for them.

Furthermore, Porteus confirms that it was not until 2005 – over two full years after the priority date of the instant application – that zinc finger nucleases were actually shown to cleave endogenous targets. (See, e.g., Porteus, section bridging pages 970-971 discussing Urnov et al. (2005) *Nature* 435:646-651).

Even after Urnov's publication, Porteus still teaches that, as of 2005, the ZFN-mediated cleavage of endogenous targets was not predictable (Porteus, page 971, right column):

> Future work will be needed to translate these *in vitro* findings to *in vivo* applications and to determine whether zinc finger nucleases create undesired genomic instability.

Clearly then, the evidence of record, including the references themselves, establishes that well after Choulika, Bibikova and Appellants' priority date cleavage of endogenous chromosomal sites with zinc finger nucleases was not predictable. The evidence of record (including post-filing date evidence) clearly establishes that prior to Appellants' disclosure; the skilled artisan would not have found it predictable that zinc finger nucleases would bind to endogenous targets. The Examiner may wish it to be otherwise (or know it to be otherwise now based on the instant specification), but wishing and hindsight reconstruction cannot support an obviousness rejection. Thus, the rejection should be withdrawn.

CONCLUSION

For the reasons stated above, Appellants respectfully submit that the claims on appeal are in condition for allowance. Accordingly, Appellants request that the rejections of the claims on appeal be reversed, and that the application be remanded to the Examiner so that the appealed claims can proceed to allowance.

Respectfully submitted,

Date: March 31, 2009

By: _____
Dahna S. Pasternak
Registration No. 41,411
Attorney for Appellant

ROBINS & PASTERNAK LLP
1731 Embarcadero Road, Suite 230
Palo Alto, CA 94303
Tel.: (650) 493-3400
Fax: (650) 493-3440

CLAIMS APPENDIX

21. A vector comprising:

(1) a nucleic acid encoding a chimeric nuclease that comprises:

(i) a zinc finger DNA binding domain;

(ii) a cleavage domain; and

(iii) a nuclear localization signal; and

(2) a nucleic acid comprising a repair substrate that comprises:

(i) a nucleic acid sequence that is substantially identical to a region flanking a target sequence in chromosomal DNA; and

(ii) a nucleic acid sequence which replaces the target sequence upon recombination between the repair substrate and the target sequence.

28. An isolated mammalian cell comprising:

(a) a chimeric nuclease comprising a zinc finger DNA-binding domain and a cleavage domain; and

(b) a repair substrate comprising

(i) a nucleic acid sequence that is substantially identical to a region flanking a target sequence in endogenous chromosomal DNA; and

(ii) a nucleic acid sequence which replaces the target sequence upon recombination between the repair substrate and the target sequence.

99. The vector of claim 21, wherein the nucleic acid encoding the chimeric nuclease is operably linked to a promoter.

100. The vector of claim 99, wherein the promoter is an inducible promoter.

101. The vector of claim 99, wherein the vector is a viral vector.

102. The vector of claim 21, further comprising a nucleic acid encoding a second chimeric nuclease, wherein the second chimeric nuclease forms a heterodimer with said chimeric nuclease.

103. The cell of claim 28, wherein the chimeric nuclease is encoded by a nucleic acid that is operably linked to a promoter.

104. The cell of claim 103, wherein the promoter is an inducible promoter.

107. The cell of claim 28, wherein the cleavage domain comprises a cleavage domain of a type IIs restriction endonuclease.

108. The cell of claim 107, wherein the cleavage domain comprises a FokI cleavage domain.

EVIDENCE APPENDIX

The following document is attached to this Brief:

(1) Porteus et al. (2005) *Nature Biotechnology* 23:967-973, submitted as with the Response of December 11, 2008 and indicated considered by the Examiner in the Advisory Action mailed January 9, 2009 (see, e.g., paragraph 6 of Advisory Action, stating "The Examiner also acknowledges the of the Porteus et al. reference.").

RELATED PROCEEDINGS APPENDIX

As noted above on page 2 of this Appeal Brief, Appellants are not aware of any related Appeals or Interferences and, accordingly, no documents are submitted with this Appendix.

nature biotechnology

REVIEW

Gene targeting using zinc finger nucleases

Matthew H Porteus[1] & Dana Carroll[2]

The ability to achieve site-specific manipulation of the mammalian genome has widespread implications for basic and applied research. Gene targeting is a process in which a DNA molecule introduced into a cell replaces the corresponding chromosomal segment by homologous recombination, and thus presents a precise way to manipulate the genome. In the past, the application of gene targeting to mammalian cells has been limited by its low efficiency. Zinc finger nucleases (ZFNs) show promise in improving the efficiency of gene targeting by introducing DNA double-strand breaks in target genes, which then stimulate the cell's endogenous homologous recombination machinery. Recent results have shown that ZFNs can be used to create targeting frequencies of up to 20% in a human disease-causing gene. Future work will be needed to translate these *in vitro* findings to *in vivo* applications and to determine whether zinc finger nucleases create undesired genomic instability.

Knowledge of the complex interplay between the genome, the physiologic processes it governs and the environment with which it interacts has increased. Gene targeting has provided an important research tool for probing this complex interplay and for manipulating the genome. In gene targeting, an exogenously introduced DNA fragment replaces an endogenous segment of DNA by homologous recombination (**Box 1**). This process was reported in yeast more than 25 years ago and variations on this technique are now commonly used for evaluating gene function in that organism[1–3].

Gene targeting also has been demonstrated in mouse cells, and when it is applied to embryonic stem (ES) cells, it has enabled the production of mutant mice, both for studying gene function and for creating models of human genetic diseases[4,5]. Although positive selection for the integrated gene is sufficient to recover the desired yeast cells, elegant and powerful selections for the transgene and against nontargeted integration had to be devised for gene targeting in mouse cells where most of the transgenes integrate at inappropriate sites in the genome; the advances have made this technique nearly routine[6,7]. Thousands of transgenic mice and ES cell lines with precise genomic alterations have been created; their characterization has increased our understanding of mammalian physiology and the pathogenesis of numerous human diseases. Nonetheless, many applications of gene targeting are hindered by its inherently low frequency and the need for selection in culture before incorporation into whole animals.

In addition to its experimental applications, gene targeting could be useful in gene therapy. Human monogenic diseases, such as sickle cell disease, hemophilia, cystic fibrosis and Huntington disease, are potentially ideal targets for genome-based therapies. Finding a cure for such diseases would eliminate billions of dollars in healthcare costs, not to mention immeasurable family and societal costs. Current approaches to gene therapy rely largely on methods that add back a normal copy of the defective gene, typically using a viral vector as carrier. Despite limitations of this approach—immunological reaction to the virus, long-term silencing of the therapeutic gene and insertional mutagenesis—and some well-publicized setbacks[8], several promising advances have been reported[9,10].

An alternative to gene addition would be gene correction through gene targeting, which allows correction of the mutation *in situ* leaving the rest of the genome unperturbed. This strategy has several advantages over gene addition procedures, including the following: the risk of mutations arising from random insertion is reduced because the approach aims to incorporate exogenous DNA at a predetermined site in the chromosome; the exogenous DNA does not have to include a complete protein coding sequence or separate signals to ensure its expression because the donor is simply correcting a mutation in an endogenous locus; and inappropriate tissue specificity, timing, level and duration of expression are not issues because the targeted gene remains under normal, endogenous controls. Thus, if targeted correction could be accomplished with high efficiency and without significant side effects, normal function should be restored.

What limits the frequency of gene targeting? Experiments in model systems have demonstrated clearly that manipulations of the donor DNA have very modest effects, but activation of the chromosomal target can improve the frequency by several orders of magnitude. Both in yeast and in mammalian cells, making a double-strand break (DSB) in the target effectively increases its interaction with an exogenous donor DNA. This is easily understood: an intact segment of chromosome does not benefit by interacting with another DNA; but

[1]Departments of Pediatrics and Biochemistry, University of Texas Southwestern Medical Center, [2]Department of Biochemistry, University of Utah, School of Medicine. Correspondence should be addressed to M.H.P. (matthew.porteus@utsouthwestern.edu).

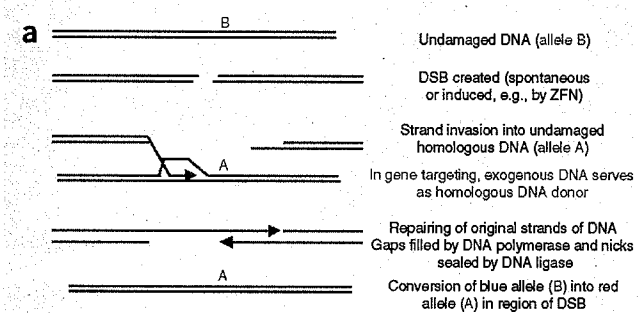
Published online 8 August 2005; doi:10.1038/nbt1125

Box 1 Homologous recombination

The maintenance of genomic integrity requires cells to repair DNA damage with high fidelity[48,49]. One of the most dangerous DNA lesions that cells encounter are DNA double-strand breaks (DSBs) because every DSB can potentially lead to cell death or to oncogenic mutations. Fortunately, cells have redundant mechanisms to repair DSBs, among them homologous recombination. Homologous recombination, which has been reviewed elsewhere[50,51] is basically a 'copy and paste' mechanism. This process uses an undamaged homologous segment of DNA, usually the sister-chromatid, as a template from which to copy the information across the break (**Fig. 1a**). Because it recovers a normal copy of the damaged DNA, homologous recombination is the most accurate form of DSB repair.

An alternative pathway of DSB repair is nonhomologous end joining, which joins ends without regard for homology and often results in small, localized deletions and/or insertions. A broken end may also become joined to a completely unrelated site resulting in a chromosomal translocation (**Fig. 1b**)

In addition to repairing accidental DSBs, cells also use homologous recombination to create regulated genomic rearrangements. During meiosis, mating type switching in yeast, and the generation of immunoglobulin and T-cell receptor diversity in certain species, the rearrangements are created by homologous recombination[52]. In controlled rearrangements, a specific nuclease creates an intentional DSB and the DSB is repaired using a DNA template other than the sister chromatid. In zinc finger nuclease-mediated gene targeting, the goal is to mimic these natural rearrangements by creating a gene-specific DSB to activate the cell's endogenous homologous recombination machinery while simultaneously providing a DNA repair donor to introduce the desired genetic changes.



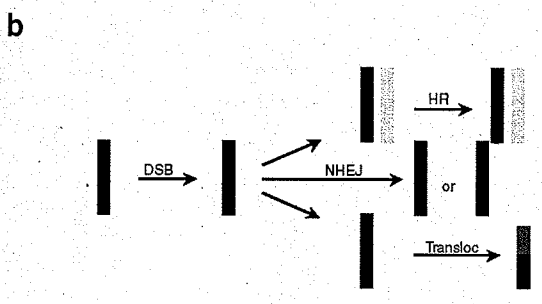


Figure 1 Double-strand break repair. (**a**) Synthesis-dependent DSB repair. This model, one of several proposed mechanisms, illustrates the essential features of DSB repair. A DSB is created in gene 'B,' which is then processed to form free 3′ single-strand tails. The homologous recombination machinery uses the free 3′ ends to invade a homologous donor. In the normal repair of a DSB, the donor is usually the sister chromatid, which is identical to the damaged allele. In gene targeting, the donor is an extrachromosomal fragment of DNA. After strand invasion, primed DNA synthesis occurs to generate an intact strand using the invading DNA as a template. The process is completed by annealing the new strand of DNA to its original partner and using that new DNA as a template for DNA synthesis. Allele 'B' is converted into allele 'A' whereas allele 'A' is unchanged. (**b**) Three outcomes of DSB repair. Dark and light blue lines represent related chromosomes that allow repair by homologous recombination. Nonhomologous end joining (NHEJ) often repairs a DSB cleanly, in a nonmutagenic fashion, but it can also result in a mutation creating a novel sequence at the junction (shown as a checkered box). The red chromosome is unrelated to the blue one, so the joining event causes a translocation.

a DSB is potentially lethal damage that must be repaired, and one pathway of repair is by homologous recombination with a closely related sequence (**Box 1** and **Fig. 1**).

Jasin and colleagues pioneered the use of highly specific DNA cleavage to investigate the stimulation of homologous recombination by DSBs in mammalian cells, making use of the yeast enzyme I-SceI encoded by *SceI*[11,12]. *SceI* is a member of the homing endonuclease family of genes, so-called because they catalyze their own duplication into alleles by creating site-specific DSBs, which then initiate their own transfer by homologous recombination; it cuts DNA at an 18 base pair (bp)-long recognition site. When a *SceI* recognition site is inserted into a target gene and SceI is expressed in the cell, homologous recombination and gene targeting are stimulated by over 1,000-fold[13,14]. The stimulation of gene targeting by *SceI* has been accomplished in several cell lines, including mouse ES cells, indicating that DSB-induced homologous recombination is a universal cellular phenomenon[15–18]. Under optimized conditions, targeting rates of 3–5% have been achieved using a reporter gene[19].

This work highlights the power of a DSB in stimulating gene targeting to levels that would be experimentally and therapeutically useful. To harness the stimulatory power of DSBs requires a method for creating site-specific DSBs in endogenous genes. There are several ways to achieve this using modified triplex-forming oligonucleotides[20], modified polyamides[21,22], modified peptide-nucleic acids[23], modified homing endonucleases or zinc finger nucleases (ZFNs) (**Box 2**). Some success has been achieved targeting genes with modified homing endonucleases and this is covered in **Box 3**. In this review we focus on recent progress made with ZFNs. ZFNs are artificial fusion proteins that link a zinc finger DNA binding domain to a nonspecific nuclease domain. Results in model organisms indicate that ZFNs will be effective in producing designed mutations for genetic studies, and the first studies in human cells encourage pursuit of ZFNs for potential use in human gene therapy.

Development of ZFNs

ZFNs (originally termed chimeric restriction enzymes) were first developed by Chandrasegaran and coworkers. (The history of the initial development of ZFNs and other hybrid nucleases has been reviewed elsewhere[24–26].) They hypothesized that they could create novel sequence specificities by fusing the nonsequence-specific cleavage domain of the *FokI* type II restriction endonuclease (Fn domain) to a new DNA-binding domain. First with a *Drosophila melanogaster* homeobox domain, then with a zinc finger-DNA binding domain and finally with the yeast

Box 2 Zinc finger basics

The protein modules known as C_2H_2 zinc fingers (ZFs), originally discovered by Klug and coworkers in 1986 (ref. 53), are found in the DNA-binding domain of the most abundant family of transcription factors in most eukaryotic genomes. The human genome contains at least 4,000 such domains in over 700 proteins, which represents ~2% of human genes[41,47]. As illustrated in **Figure 2**, each finger is composed of 30 amino-acids, folds into a ββα configuration, coordinates one Zn^+ atom using two cysteines and two histidine residues, and contacts primarily 3 bps of DNA[54]. Two critical features of the structure are that each finger binds its 3-bp target site independently and that each nucleotide seemed to be contacted by a single amino acid side chain projecting from one end of the α-helix into the major groove of the DNA. From these features, two predictions were made. The first is that by combining individual zinc fingers with different triplet targets, the overall binding specificity of the zinc finger protein could be changed. The second is that by altering individual amino acid residues in the α-helix, the specificity for an individual finger could be altered. These critical predictions have been substantiated in some contexts by a number of different labs and are reviewed elsewhere[32,55–57]. Individual fingers have been designed to recognize many of the 64 different target triplets, but the greatest success has been in designing zinc fingers to recognize 5′-GNN-3′ triplets (where N represents any of the four bases)[58,59]. Although zinc finger recognition codes have been proposed[32,55,60], no code currently exists that consistently results in zinc fingers with high affinity binding. Improving the specificity of ZF binding, such as by increasing the number of fingers or by constructing multifinger proteins using two-finger units, remains an active area of research[33,41,46,57,61–63].

Gal4 DNA-binding domain, they demonstrated that cutting could be redirected in the chimeras[24,27,28]. ZFNs (**Fig. 2**) consist of an N-terminal zinc finger DNA-binding domain, a variable peptide linker and a C-terminal Fn domain (**Box 2**). Whereas initial *in vitro* work suggested that a ZFN could cleave DNA at a monomeric copy of its recognition site[29], subsequent studies, demonstrated that ZFNs cleave as dimers[30,31].

Using a *Xenopus laevis* oocyte system, Bibikova *et al.*[31] showed that the most efficient cleavage and recombination was obtained when the binding sites were inversely oriented and separated by six nucleotides, and when there was no intentional linker between the zinc finger and nuclease domains. Taken together, these experiments showed that ZFNs not only could create DSBs on naked DNA templates *in vitro*, but also create DSBs in a cell, thereby activating substrates for homologous recombination.

The *in vitro* and *X. laevis* studies were done using zinc finger DNA binding domains with known recognition sites. The appeal of ZFNs, however, is that the zinc finger DNA binding domain could be modified to recognize novel target sequences, including those in endogenous genes[32–34] (**Box 2**).

ZFNs in model organisms

The first genomic locus to be targeted successfully with designed ZFNs was the yellow gene of the fruit fly *D. melanogaster*[35]. Bibikova *et al.*[35] produced a pair of three-finger ZFNs for a sequence within this gene based on the fact that fingers had been identified at that time that would bind all DNA triplets of the form 5′-GNN-3′. Given the requirements for ZFN cleavage, they selected a site with the form, 5′-NNC NNC NNC NNNNNN GNN GNN GNN-3′ (5′-$(NNC)_3N_6(GNN)_3$-3′). Two zinc finger nucleases were assembled to recognize one 9-bp site each. Heat-shock induction of these two proteins from integrated transgenes in fly larvae led to both targeted mutagenesis following cleavage[35] and, in the presence of a marked donor DNA, targeted gene replacement by homologous recombination[36]. These alterations were stably passed through the germ line and, in initial studies, represented a few percent of all the chromosomal targets. Recent experiments have extended this procedure to two additional *D. melanogaster* loci, and targeting frequencies up to 25% have been achieved (D.C. *et al.*, unpublished data).

This approach should be applicable to essentially all model organisms, although unique experimental conditions will likely have to be established for each organism. Evidence to support this broad applicability comes from experiments showing that ZFNs create targeted mutations in the plant *Arabidopsis thaliana*[37]. These studies indicate that ZFNs will be powerful tools for making directed modifications in experimental organisms for functional studies and for creating models of human genetic diseases.

Box 3 Modified homing endonucleases

Homing endonucleases, such as I-SceI of yeast, are natural genetic elements that catalyze their own duplication into recipient alleles by creating site-specific DSBs that initiate their own genetic transfer by homologous recombination[64–66]. A key feature of these enzymes is that they create DSBs at recognition sites that are 14- to 40-bp long[64]. A second key feature is that their expression in mammalian cells does not cause overt cytotoxicity or seem to cause gross chromosomal rearrangements[13]. This appealing attribute is at least in part due to their site specificity. Although several hundred different homing endonucleases with different recognition sites have been identified, the major limitation to using them in gene targeting is that most mammalian genes do not have recognition sites for them. One strategy, therefore, is to use protein engineering to modify homing endonucleases to recognize target sites in mammalian genes.

This line of experimentation is still in its infancy but several investigators have made progress using structure-based protein engineering. In this work, chimeric homing endonucleases have been made with novel recognition sites, and *in vitro* modifications have been made that alter the target site specificity[67–70]. This work suggests that these enzymes can be modified to recognize new recognition sites. Currently, however, no modified homing endonuclease has been made that recognizes a sequence from an endogenous mammalian gene. Moreover, because one of their attractions is their lack of cytotoxicity, it also remains to be seen whether modified versions retain that characteristic or if by changing the site-specificity one also loosens their site specificity, thereby creating additional DSBs at undesired sites and consequent cytotoxicity.

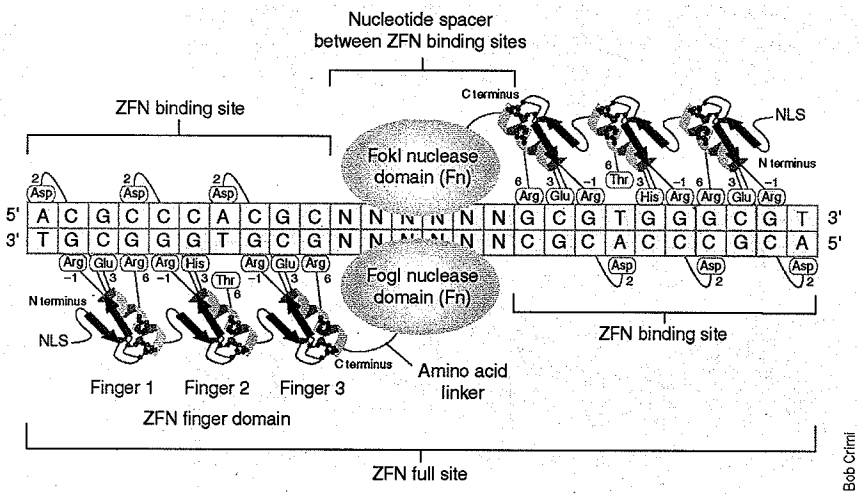


Figure 2 ZFN homodimer binding to DNA. Shown is a three-finger zinc finger linked to the Fn domain through a flexible peptide linker. At the N-terminus of each ZFN resides a nuclear localization signal (NLS). The Fn domain is linked to the C-terminal finger (in this case finger 3) of the zinc finger domain. For most efficient cleavage there is no amino acid linker between the zinc finger domain and the Fn domain. The binding sites are arranged in an inverted orientation so that one ZFN is making most of its major contacts with one strand of DNA, whereas the other ZFN is making most of its major contacts with the other strand of DNA. Between the two binding sites is a nucleotide spacer (NNN...), the sequence of which does not seem to be important. This figure is a modification of Figure 2c from Jantz *et al.*[47].

ZFNs in human somatic cells

The first demonstration that ZFNs could stimulate gene targeting in mammalian somatic cells came from Porteus (M.H.P.) and Baltimore[19]. In these experiments, recognition sites for known zinc finger DNA-binding domains were inserted into a green fluorescent protein (GFP) reporter gene, which was integrated as a single copy into the genome of the human embryonic kidney cell line (HEK293; Fig. 3). In this system, gene targeting is measured by the correction of an integrated mutant GFP target gene by a transfected donor plasmid and the resultant conversion of GFP-negative cells into GFP-positive cells. ZFNs were made using a three-finger zinc finger domain from a natural zinc finger protein, Zif268 (Zif-ZFN) and a designed three-finger domain, QQR (QQR-ZFN)[30,31]. The stimulation of targeting was most efficient when the ZFN recognition sites were oriented as inverted repeats separated by six nucleotides and the ZFN did not contain a linker between the zinc finger and the Fn domains, showing that the same rules applied to mammalian cells as had been found earlier with *X. laevis* oocytes[19,30,31]. This work and the work of Bibikova *et al.* (2003) has been briefly reviewed elsewhere[38].

A critical next step was to design ZFNs to recognize natural sequences and demonstrate that they could also stimulate targeting in mammalian cells as had been done in the *D. melanogaster* germ line[36]. The prediction that sites of the form 5′-$(GNN)_3$-3′ could be targeted in mammalian cells with assembled three-finger proteins was verified when ZFNs to several different targets were shown to stimulate gene targeting[39]. In addition, this work showed that targeting with ZFN could be induced simultaneously at both the site of the break and at a distance of 400 bp from the break, demonstrating that a single pair of ZFNs can stimulate targeting in a relatively large region surrounding a DSB.

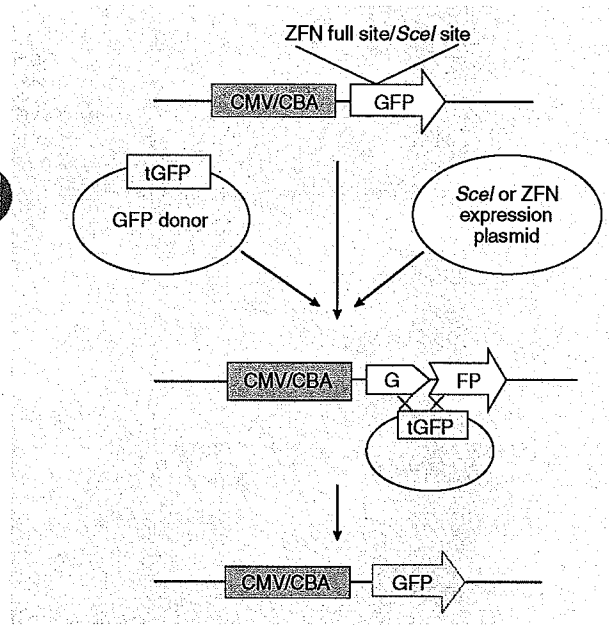


Figure 3 GFP gene-targeting reporter system. A mutated GFP gene, with an inserted in-frame stop codon and recognition sites for *SceI* and zinc finger nuclease, is integrated as a single copy into the genome of a cell (top line). The cell is transfected with a plasmid with a nonfunctional GFP gene (tGFP) and expression plasmids for either the ZFNs or *SceI*. The integrated GFP is repaired by gene targeting, and the cell becomes GFP positive. CMV/CBA, cytomegalovirus enhancer/chicken β-actin hybrid promoter; GFP, green fluorescent protein.

ZFN-mediated gene targeting of the SCID gene

Recently, a paper by Urnov *et al.*[40] (M.H.P. contributed to this work) has reported that designed ZFNs can cleave an endogenous human gene in cultured cells and lead to targeted gene replacement in up to 20% of the cells. The target was the gene for interleukin (IL)-2Rγ, a cytokine receptor that is required for T-cell development and the establishment of a functional immune system[41]. Mutations in the human IL2RG gene (γ_c) are the most common cause of severe combined immunodeficiency (SCID) and, importantly, it has been shown to be an effective target for gene addition therapy. In the earlier experiments, a viral vector was used to deliver a normal IL-2Rγ gene to bone marrow cells isolated from affected children[8,9]. Returning these manipulated cells to their hosts resulted in restoration of immune competence. In three cases, however, T-cell leukemias arose owing to activation of an oncogene as a result of nearby integration of the therapeutic transgene[10]. This highlights the need for procedural adjustments, but the clinical success is very encouraging.

Urnov *et al.*[40] used a commercial archive of two-finger modules[42] to create two four-finger ZFNs (γ_c-ZFN-L and γ_c-ZFN-R) targeted to exon 5 of the γ_c gene, a mutational hot spot on the gene. In addition to the novel source of zinc fingers, the binding sites in this case were separated by only 5 bp, which further expands the repertoire of sites that

Table 1 Potential applications of zinc finger nucleases

Experimental uses	Drug development	Therapeutics
Create knockout genes (cell lines, primary cells, transgenic animals)	Create humanized cell lines	Correction of genes in monogenic diseases (e.g., Huntington disease)
Create point mutations or small deletions in permanent or primary cell lines	Create cell lines for drug target validation	Inserting genes into precise (safe and permissive) locations for correcting complex mutations (hemophilia A) and introducing RNAi, for example
Improve efficiency of gene targeting in ES cells	Create cell lines for high-throughput screening for novel compounds	Altering alleles; for example, the CCR5 gene to create resistance to HIV.
Create targeted transgenics with insertions into precise genomic locations		Designer immunotherapeutics
Genome manipulation in model organisms currently without gene targeting mechanism (worms, zebrafish)		Modification of stem cells

ZFNs target. After showing that the γ_c ZFNs had a high affinity binding to their target sites *in vitro*, we then tested them using a GFP reporter system and found that they efficiently stimulated targeting. Subsequent protein engineering optimized binding of each ZFN to its cognate site, leading to a fivefold improvement in targeting in the GFP reporter system. Importantly, in K562 cells, a transformed human erythroleukemia cell line, the γ_c ZFNs stimulated mono-allelic targeting in 11% of cells and bi-allelic targeting in 6% of cells without selection.

Furthermore, successful targeting was achieved in cultured primary T cells and in established lines, and conversion both from normal to mutant and back to normal was demonstrated in successive experiments. These experiments gave clear proof of principle that ZFNs could be used as a powerful tool to create subtle and specific changes in the genome of human somatic cells and that ZFNs could be used to correct mutations that cause human disease.

Toxicity and other undesired effects

In early studies, it was noticed that expression of ZFNs could have cytotoxic effects. In *D. melanogaster* for example, one of the two ZFNs designed for the yellow gene proved to be lethal when overexpressed, although tolerable and effective levels of expression were readily found[35]. In the initial experiments with human cells[19] toxicity was demonstrated by loss of targeted GFP$^+$ cells upon continued culture. The four-finger proteins constructed by Urnov *et al.*[40] for the human γ_c gene, in contrast, did not show toxicity. Using a relatively insensitive assay, they did not detect any gross chromosomal rearrangements, although this does not rule out the possibility of a low frequency of translocation.

With *D. melanogaster*, the lethality was demonstrated to be a consequence of excessive cleavage, as a point mutation in the nuclease active site restored full viability (K. Beumer and D.C., unpublished data). We assume that the toxicity in mammalian cells is also due to cleavage of nontarget sequences; when the number of 'off-target' DSBs becomes too great for that cell type, cell death ensues. An advantage of ZFNs is that dimerization of the nuclease domain is required for cleavage. Thus, a pair of three-finger ZFNs will usually have a unique 18-bp site at which it most efficiently cuts. Off-target cleavage is likely the result of two ZFNs binding at noncanonical sites, perhaps ones related in sequence to the desired target or may be the result of binding of a single ZFN with solution dimerization of the nuclease domain as can occur with natural FokI *in vitro*[43]. Evidence from experiments with both cultured cells[40] and flies (D.C. *et al.*, unpublished observations) shows that it is possible to design ZFNs with sufficient specificity to reduce toxicity, but new approaches may also be required.

Another consequence of cleavage by ZFNs is the creation of break-induced sequence alterations through nonhomologous end joining (**Box 1** and **Fig. 1**). Mutations of this sort can occur both at the desired target if the DSB is repaired by nonhomologous end-joining rather than by homologous recombination with the donor and at sites of off-target cleavage. In some instances the goal may be to alter the target sequence—for example, to knock out the activity of a particular gene—but in directed gene targeting procedures, particularly in gene therapy settings, it would be undesirable to create new mutations while correcting an existing one. Finally, it is known that DSBs are a source of oncogenic translocations[44,45]. It is important, therefore, to determine if ZFNs are creating such translocations by the induction of DSBs and to develop assays to detect these rare but potentially dangerous events.

Future directions

The development of ZFNs to stimulate gene targeting by homologous recombination in mammalian somatic cells represents the synergistic fusion of two seemingly independent fields: the study of zinc finger domains and the study of homologous recombination. The results reported here provide cause for optimism that ZFN-mediated targeting will provide a useful experimental tool for manipulating the mammalian genome for many of the potential experimental applications highlighted in **Table 1**. Moreover, with further development, the ZFN strategy may be applied in the treatment of human genetic diseases as well as in other areas of biotechnology. Before such applications can be realized and ZFN approaches become widely adopted, however, several challenges remain: first, applicability of the ZFN approach needs to be broadened; second, the method for delivering ZFNs and repair substrate to cells requires optimization; and third, our understanding of the process of homologous recombination itself needs to be enhanced.

Efforts to broaden the applicability of the approach will require not only the design of ZFNs targeting a greater variety of gene targets but also the determination of the conditions for performing gene targeting in different cell types. Until now, ZFNs have been primarily applied to transformed mammalian cell lines that are relatively resistant to apoptotic stimuli. An important advance, therefore, will be to develop ways of using ZFNs in primary cells that are more sensitive to DNA damage.

The optimization of ZFN design must address two key, related issues: specificity and cytotoxicity. Addressing these issues will necessitate thorough analysis of the mechanisms of gene target recognition and binding for the zinc finger component. Simply assembling ZFNs for sites composed entirely of GNN triplets has its limitations, but may prove to be practical for many applications. In the case of ZFNs designed to target the γ_c gene[40], the method used to create the ZFN affected both the ratio of specificity to cytotoxicity and the ZFNs and the ability to

efficiently target sequences that contain non-GNN triplets. It is not clear how many zinc fingers are optimal for activity and precise targeting. Whereas four-finger γ_c ZFNs are less cytotoxic than three-finger ZFNs in transformed mammalian somatic cells, even these show potential 'off-target' effects when expressed alone at high levels using a relatively crude cytotoxicity assay (M.H.P, unpublished data.

Several lines of investigation may offer solutions to ZFN cytotoxicity issues. First, zinc fingers may be further refined to better discriminate between the gene target of choice and related off-target binding sites. This will require continued basic research into the nature of the recognition process—an area that has been studied by structural biologists for several decades and is not trivial. Second, further research is required to elucidate the optimal number of fingers in each ZFN. Future studies should determine whether increasing the number of fingers consistently results in improved ZFNs, both from a specificity and kinetic standpoint. Experiments thus far have demonstrated that moving from three to four fingers provides significant improvement, but it is not clear whether further increases may be beneficial[46,47]. Third, efforts should focus on tighter control of the level and duration of expression of potentially toxic ZFNs. The loss of targeted cells with time in culture is likely a consequence of continued, although still transient, expression. Because the homologous recombination event is expected to occur rapidly after the target is cut, brief expression of ZFNs should be adequate. Finally, it may be possible to engineer other parts of the ZFNs—the peptide linker and the cleavage domain (including the possibility of alternative nuclease domains)—to optimize the ratio of target to off-target events.

The second main challenge for wide adoption of the ZFN approach will be to optimize the delivery method of the ZFNs and repair substrate to stem cells. Studies of ZFNs have been primarily performed using transfection techniques specific to cultured cell lines. Whether those techniques will work in primary cells or whether other methods, including the adaptation of viral delivery methods or direct microinjection as was done with *X. laevis* oocytes, will be better are important areas of future study.

A final challenge will be to increase our understanding of the process of homologous recombination itself in somatic stem cells as these are the cells that are most likely to generate long-term therapeutic benefits if targeted. All evidence suggests that homologous recombination is a universal process. However, studies are needed to analyze the rate of homologous recombination in somatic stem cells, particularly quiescent ones.

ACKNOWLEDGMENTS

We thank Scott Cameron, Jim Amatruda, Brian Cauff, Shondra Pruett, Patrick Connelly, Michael Holmes and Philip Gregory for reading the manuscript and for their helpful comments. The work in the Porteus lab is supported by the Burroughs-Wellcome Fund, a K08 award from the National Heart and Blood Institute, and UT Southwestern Medical Center. Work in the Carroll lab is supported by research grants from the US Public Health Service.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

Published online at http://www.nature.com/naturebiotechnology/

1. Hinnen, A., Hicks, J.B. & Fink, G.R. Transformation of yeast. *Proc. Natl. Acad. Sci. USA* **75**, 1929–1933 (1978).
2. Rothstein, R.J. One-step gene disruption in yeast. *Methods Enzymol.* **101**, 202–211 (1983).
3. Orr-Weaver, T.L., Szostak, J.W. & Rothstein, R.J. Yeast transformation: a model system for the study of recombination. *Proc. Natl. Acad. Sci. USA* **78**, 6354–6358 (1981).
4. Capecchi, M. Altering the genome by homologous recombination. *Science* **244**, 1288–1292 (1989).
5. Bradley, A., Ramirez-Solis, R., Zheng, H., Hasty, P. & Davis, A. Genetic manipulation of the mouse via gene targeting in embryonic stem cells. *Ciba Found. Symp.* **165**, 256–269 (1992).
6. Mansour, S.L., Thomas, K.R. & Capecchi, M.R. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* **336**, 348–352 (1988).
7. Sedivy, J.M. & Sharp, P.A. Positive genetic selection for gene disruption in mammalian cells by homologous recombination. *Proc. Natl. Acad. Sci. USA* **86**, 227–231 (1989).
8. Hacein-Bey-Abina, S. *et al.* LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415–419 (2003).
9. Cavazzana-Calvo, M. *et al.* Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**, 669–672 (2000).
10. Gaspar, H.B. *et al.* Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* **364**, 2181–2187 (2004).
11. Jasin, M. Genetic manipulation of genomes with rare-cutting endonucleases. *Trends Genet.* **12**, 224–228 (1996).
12. Rouet, P., Smih, F. & Jasin, M. Introduction of double-strand breaks into the genome of mouse cells by expression of rare-cutting endonuclease. *Mol. Cell. Biol.* **14**, 8096–8106 (1994).
13. Rouet, P., Smih, F. & Jasin, M. Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc. Natl. Acad. Sci. USA* **91**, 6064–6068 (1994).
14. Smih, F., Rouet, P., Romanienko, P.J. & Jasin, M. Double-strand breaks at the target locus stimulate gene targeting in embryonic stem cells. *Nucleic Acids Res.* **23**, 5012–5019 (1995).
15. Choulika, A., Perrin, A., Dujon, B. & Nicolas, J.-F. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of Saccaromyces cerevisiae. *Mol. Cell. Biol.* **15**, 1968–1973 (1995).
16. Sargent, R.G., Brenneman, M.A. & Wilson, J.H. Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. *Mol. Cell. Biol.* **17**, 267–277 (1997).
17. Donoho, G., Jasin, M. & Berg, P. Analysis of gene targeting and intrachromosomal homologous recombination stimulated by genomic double-strand breaks in mouse embryonic stem cells. *Mol. Cell. Biol.* **18**, 4070–4078 (1998).
18. Cohen-Tannoudji, M. *et al.* I-SceI-induced gene replacement at a natural locus in embryonic stem cells. *Mol. Cell. Biol.* **18**, 1444–1448 (1998).
19. Porteus, M.H. & Baltimore, D. Chimeric nucleases stimulate gene targeting in human cells. *Science* **300**, 763 (2003).
20. Kuan, J.Y. & Glazer, P.M. Targeted gene modification using triplex-forming oligonucleotides. *Methods Mol. Biol.* **262**, 173–194 (2004).
21. Wurtz, N.R. & Dervan, P.B. Sequence specific alkylation of DNA by hairpin pyrrole-imidazole polyamide conjugates. *Chem. Biol.* **7**, 153–161 (2000).
22. Dervan, P.B. & Edelson, B.S. Recognition of the DNA minor groove by pyrrole-imidazole polyamides. *Curr. Opin. Struct. Biol.* **13**, 284–299 (2003).
23. Kaihatsu, K., Janowski, B.A. & Corey, D.R. Recognition of chromosomal DNA by PNAs. *Chem. Biol.* **11**, 749–758 (2004).
24. Kim, Y.G., Cha, J. & Chandrasegaran, S. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc. Natl. Acad. Sci. USA* **93**, 1156–1160 (1996).
25. Chandrasegaran, S. & Smith, J. Chimeric restriction enzymes: what is next? *Biol. Chem.* **380**, 841–848 (1999).
26. Kandavelou, K., Mani, M., Durai, S. & Chandrasegaran, S. in *Nucleic Acids and Molecular Biology*, vol. 14 (ed. Pingoud, A.M.) 413–434 (Springer-Verlag, Heidelberg, Germany, 2004).
27. Kim, Y.G. & Chandrasegaran, S. Chimeric restriction endonuclease. *Proc. Natl. Acad. Sci. USA* **91**, 883–887 (1994).
28. Kim, Y.G., Smith, J., Durgesha, M. & Chandrasegaran, S. Chimeric restriction enzyme: Gal4 fusion to FokI cleavage domain. *Biol. Chem.* **379**, 489–495 (1998).
29. Smith, J., Berg, J.M. & Chandrasegaran, S. A detailed study of the substrate specificity of a chimeric restriction enzyme. *Nucleic Acids Res.* **27**, 674–681 (1999).
30. Smith, J. *et al.* Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. *Nucleic Acids Res.* **28**, 3361–3369 (2000).
31. Bibikova, M. *et al.* Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. *Mol. Cell. Biol.* **21**, 289–297 (2001).
32. Pabo, C.O., Peisach, E. & Grant, R.A. Design and selection of novel Cys2His2 zinc finger proteins. *Annu. Rev. Biochem.* **70**, 313–340 (2001).
33. Beerli, R.R. & Barbas, C.F. III. Engineering polydactyl zinc-finger transcription factors. *Nat. Biotechnol.* **20**, 135–141 (2002).
34. Isalan, M. & Choo, Y. Engineering nucleic acid-binding proteins by phage display. *Methods Mol. Biol.* **148**, 417–429 (2001).
35. Bibikova, M., Golic, M., Golic, K.G. & Carroll, D. Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. *Genetics* **161**, 1169–1175 (2002).
36. Bibikova, M., Beumer, K., Trautman, J.K. & Carroll, D. Enhancing gene targeting with designed zinc finger nucleases. *Science* **300**, 764 (2003).
37. Lloyd, A., Plaisier, C.L., Carroll, D. & Drews, G.N. Targeted mutagenesis using zinc-finger nucleases in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **102**, 2232–2237 (2005).
38. Wilson, J.H. Pointing fingers at the limiting step in gene targeting. *Nat. Biotechnol.* **21**, 759–760 (2003).
39. Porteus, M.H. in Abstract 709 presented at the Annual Meeting of the American Society of Gene Therapy, Minneapolis, Minnesota, June, 2004.

40. Urnov, F.D. *et al.* Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**, 646–651 (2005).
41. Notarangelo, L.D. *et al.* Of genes and phenotypes: the immunological and molecular spectrum of combined immune deficiency. Defects of the gamma(c)-JAK3 signaling pathway as a model. *Immunol. Rev.* **178**, 39–48 (2000).
42. Jamieson, A.C., Miller, J.C. & Pabo, C.O. Drug discovery with engineered zinc-finger proteins. *Nat. Rev. Drug Discov.* **2**, 361–368 (2003).
43. Bitinaite, J., Wah, D.A., Aggarwal, A.K. & Schildkraut, I. FokI dimerization is required for DNA cleavage. *Proc. Natl. Acad. Sci. USA* **95**, 10570–10575 (1998).
44. Richardson, C. & Jasin, M. Frequent chromosomal translocations induced by DNA double-strand breaks. *Nature* **405**, 697–700 (2000).
45. Elliott, B. & Jasin, M. Double-strand breaks and translocations in cancer. *Cell. Mol. Life Sci.* **59**, 373–385 (2002).
46. Tan, S. *et al.* Zinc-finger protein-targeted gene regulation: genomewide single-gene specificity. *Proc. Natl. Acad. Sci. USA* **100**, 11997–12002 (2003).
47. Jantz, D., Amann, B.T., Gatto, G.J. Jr. & Berg, J.M. The design of functional DNA-binding proteins based on zinc finger domains. *Chem. Rev.* **104**, 789–799 (2004).
48. van Gent, D.C., Hoeijmakers, J.H. & Kanaar, R. Chromosomal stability and the DNA double-stranded break connection. *Nat. Rev. Genet.* **2**, 196–206 (2001).
49. Jasin, M. Chromosome breaks and genomic instability. *Cancer Invest.* **18**, 78–86 (2000).
50. Paques, F. & Haber, J.E. Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **63**, 349–404 (1999).
51. West, S.C. *et al.* Double-strand break repair in human cells. *Cold Spring Harb. Symp. Quant. Biol.* **65**, 315–321 (2000).
52. McCormack, W.T., Tjoelker, L.W. & Thompson, C.B. Avian B-cell development: generation of an immunoglobulin repertoire by gene conversion. *Annu. Rev. Immunol.* **9**, 219–241 (1991).
53. Diakun, G.P., Fairall, L. & Klug, A. EXAFS study of the zinc-binding sites in the protein transcription factor IIIA. *Nature* **324**, 698–699 (1986).
54. Pavletich, N.P. & Pabo, C.O. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. *Science* **252**, 809–817 (1991).
55. Wolfe, S.A., Nekludova, L. & Pabo, C.O. DNA recognition by Cys2His2 zinc finger proteins. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 183–212 (2000).
56. Segal, D.J. & Barbas, C.F. 3rd Custom DNA-binding proteins come of age: polydactyl zinc-finger proteins. *Curr. Opin. Biotechnol.* **12**, 632–637 (2001).
57. Segal, D.J. *et al.* Evaluation of a modular strategy for the construction of novel polydactyl zinc finger DNA-binding proteins. *Biochemistry* **42**, 2137–2148 (2003).
58. Liu, Q., Xia, Z., Zhong, X. & Case, C.C. Validated zinc finger protein designs for all 16 GNN DNA triplet targets. *J. Biol. Chem.* **277**, 3850–3856 (2002).
59. Segal, D.J., Dreier, B., Beerli, R.R. & Barbas, C.F. III. Toward controlling gene expression at will: selection and design of zinc finger domains recognizing each of the 5′-GNN-3′ DNA target sequences. *Proc. Natl. Acad. Sci. USA* **96**, 2758–2763 (1999).
60. Sera, T. & Uranga, C. Rational design of artificial zinc-finger proteins using a non-degenerate recognition code table. *Biochemistry* **41**, 7074–7081 (2002).
61. Kim, J.S. & Pabo, C.O. Getting a handhold on DNA: design of poly-zinc finger proteins with femtomolar dissociation constants. *Proc. Natl. Acad. Sci. USA* **95**, 2812–2817 (1998).
62. Liu, Q., Segal, D.J., Ghiara, J.B. & Barbas, C.F. III. Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. *Proc. Natl. Acad. Sci. USA* **94**, 5525–5530 (1997).
63. Moore, M., Klug, A. & Choo, Y. Improved DNA binding specificity from polyzinc finger peptides by using strings of two-finger units. *Proc. Natl. Acad. Sci. USA* **98**, 1437–1441 (2001).
64. Chevalier, B.S. & Stoddard, B.L. Homing endonucleases: structural and functional insight into the catalysts of intron/intein mobility. *Nucleic Acids Res.* **29**, 3757–3774 (2001).
65. Gimble, F.S. Invasion of a multitude of genetic niches by mobile endonuclease genes. *FEMS Microbiol. Lett.* **185**, 99–107 (2000).
66. Belfort, M. & Roberts, R.J. Homing endonucleases: keeping the house in order. *Nucleic Acids Res.* **25**, 3379–3388 (1997).
67. Chevalier, B.S. *et al.* Design, activity, and structure of a highly specific artificial endonuclease. *Mol. Cell* **10**, 895–905 (2002).
68. Epinat, J.C. *et al.* A novel engineered meganuclease induces homologous recombination in yeast and mammalian cells. *Nucleic Acids Res.* **31**, 2952–2962 (2003).
69. Sussman, D. *et al.* Isolation and characterization of new homing endonuclease specificities at individual target site positions. *J. Mol. Biol.* **342**, 31–41 (2004).
70. Gimble, F.S., Moure, C.M. & Posey, K.L. Assessing the plasticity of DNA target site recognition of the PI-SceI homing endonuclease using a bacterial two-hybrid selection system. *J. Mol. Biol.* **334**, 993–1008 (2003).